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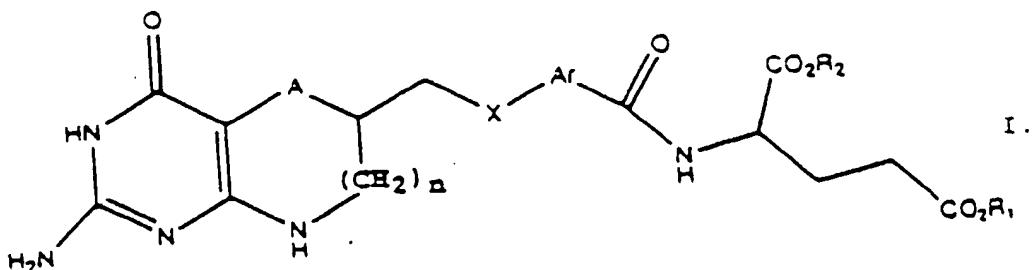
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(54) Condensed heterocyclic glutamic acid derivatives as antiproliferative agents

(57) Novel compounds of formula (I)



wherein

n is 0 to 2;

A is sulfur, CH₂, oxygen, -NH- or selenium, provided that when n is 0, A cannot be CH₂, and when n is 1, A cannot be CH₂ or NH;X is at least one substituted or unsubstituted C₁-C₃ alkyl or alkenyl group,a C₂-C₃ alkynyl group, a substituted or unsubstituted amino group, sulfur or oxygen;

Ar is a substituted or unsubstituted monocyclic carbocyclic or heterocyclic ring or fused or nonfused carbocyclic or heterocyclic ring system; and

R₁ and R₂ are independently hydrogen or a moiety which forms a readily hydrolyzable ester group or a pharmaceutically acceptable salt thereof

are found to inhibit the enzyme glycaminide ribonucleotide formyl transferase (GARFT).

A novel method of preparing such compounds is also disclosed, as well as methods and compositions for employing

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the compounds as antiproliferative agents.

Description

[0001] The present invention relates to certain compounds, defined below, which inhibit the enzyme glycinamide ribonucleotide formyl transferase (GARFT), to intermediate compounds, to pharmaceutical compositions containing the certain compounds, to the use thereof to inhibit GARFT, and to the use thereof to inhibit the growth and proliferation of the cells of higher organisms or microorganisms such as bacteria, yeast and fungi. Such effects include antitumor, antiinflammatory, antipsoriatic and immunosuppressive activity. A process for the preparation of these compounds is also disclosed.

[0002] The large class of antiproliferative agents includes antimetabolite compounds. A particular subclass of antimetabolites known as antifolates or antifolates are antagonists of the vitamin folic acid. Typically, antifolates closely resemble the structure of folic acid and incorporate the characteristic P-benzoyl glutamate moiety of folic acid. The glutamate moiety of folic acid takes on a double negative charge at physiological pH. Therefore, this compound and its analogs have an active energy driven transport system to cross the cell membrane and exert a metabolic effect.

[0003] Glycinamide ribonucleotide formyl transferase (GARFT) is a folate dependent enzyme in the *de novo* purine biosynthesis pathway. This pathway is critical to cell division and proliferation. Shutting down this pathway is known to have an antiproliferative effect, in particular, an antitumor effect. Thus, a number of folate analogs have been synthesized and studied for their ability to inhibit GARFT. A prototypic specific tight binding inhibitor of GARFT, 5,10-didezatetrahydrofolic acid, has been reported to show antitumor activity. See F.M. Muggia, "Folate antimetabolites inhibitory to *de novo* purine synthesis" in New Drugs, Concepts and Results in Cancer Chemotherapy, pp. 65-87, Kluwer Academic Publishers, Boston (1992).

[0004] The present invention introduces a novel class of compounds containing a glutamic acid or ester moiety. These compounds are effective in inhibiting the enzyme glycinamide ribonucleotide formyl transferase (GARFT) and the growth and proliferation of cells of higher organisms and of microorganisms such as bacteria, yeast and fungi. The invention further relates to pharmaceutical compositions containing these compounds or suitable salts thereof and the use of these compounds as inhibitors of the enzyme GARFT.

[0005] As stated above, compounds of the invention possess anti-proliferative activity, a property which may express itself in the form of anti-tumor activity. A compound of the invention may be active *per se*, or it may be a precursor which is converted *in vivo* to an active compound. Preferred compounds of the invention are active in inhibiting the enzyme GARFT. Particularly preferred compounds are active in inhibiting the growth of the L1210 cell line, a mouse leukemia cell line which can be grown in tissue culture. Compounds of the invention should also be active in inhibiting the growth of bacteria such as *Escherichia coli* gram negative bacteria which can be grown in culture.

[0006] The compounds according to the invention, as well as the pharmaceutically acceptable salts thereof, may be incorporated into convenient dosage forms such as capsules, tablets, or injectable preparations. Solid or liquid pharmaceutically acceptable carriers may also be employed. Solid carriers include starch, lactose, calcium sulphate dihydrate, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate and stearic acid. Liquid carriers include syrup, peanut oil, olive oil, saline solution and water.

[0007] The carrier or diluent may include any prolonged release material, such as glyceryl monostearate or glyceryl distearate, alone or with wax. When a liquid carrier is used, the preparation may be in the form of a syrup, elixir, emulsion, soft gelatin capsule, sterile injectable liquid (e.g. solution) or a nonaqueous or aqueous liquid suspension.

[0008] The pharmaceutical preparations are prepared following conventional techniques of the pharmaceutical chemist involving steps such as mixing, granulating, and compressing when necessary for tablet forms, or mixing, filling and dissolving the ingredients as appropriate to give the desired products for oral, parenteral, topical, intravaginal, intranasal, intrabronchial, intraocular, intraaural and rectal administration.

[0009] The compositions of the invention may further comprise one or more other compounds which are anti-tumor agents such as: a mitotic inhibitor (for example: vinblastine); alkylating agents; dihydrofolate reductase inhibitors or TS inhibitors; antimetabolites (for example, 5-fluorouracil and cytosine arabinoside); intercalating antibiotics (for example, adriamycin and bleomycin); enzymes (for example, asparaginase); topoisomerase inhibitors (for example, etoposide); biological response modifiers (for example, interferon); or the compounds described in copending and commonly assigned U.S. patent application no. 07/991,259, filed December 16, 1992 and incorporated specifically by reference herein.

[0010] The compositions of the invention may also comprise one or more other compounds including antibacterial, antifungal, antiparasitic, antiviral, antipsoriatic and anticoccidial agents. Exemplary antibacterial agents include, for example, sulfonamides such as sulfamethoxazole, sulfadiazine, sulfamer or sulfadoxine; dihydrofolic reductase inhibitors such as trimethoprim, bromodiaprim, or trimetrexate; penicillins; cephalosporins; aminoglycosides; bacteriostatic inhibitors of protein synthesis; the quinolone carboxylic acids and their fused isothiazolo-analogs.

[0011] Another aspect of the invention relates to a therapeutic process of inhibiting the growth and proliferation of cells of higher organisms or microorganisms which comprises administering to a host an effective amount of a compound according to the present invention. The compounds of the invention are particularly useful in the treatment of

mammalian hosts such as human hosts and in the treatment of avian hosts. A particularly preferred therapeutic process comprises administering to a host an effective amount of a compound according to the present invention to inhibit GARFT.

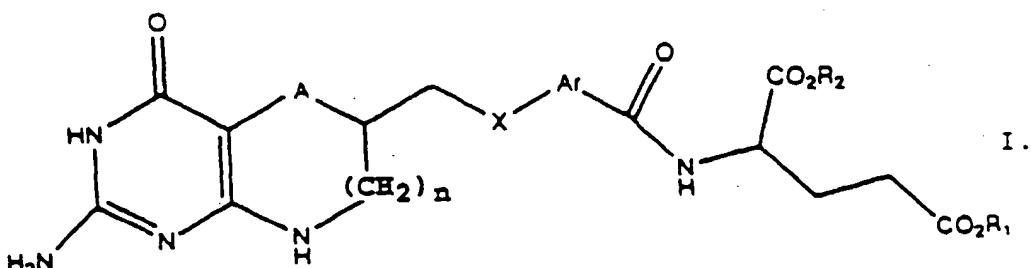
[0012] Many of the antiproliferative drugs described herein or pharmaceutically acceptable salts thereof can be employed in the therapeutic process of the invention. The compounds may be administered in the form of a pharmaceutically acceptable composition comprising a diluent or carrier such as those described above.

[0013] Doses of the compounds preferably include pharmaceutical dosage units comprising an effective quantity of the active compound. An "effective quantity" means a quantity sufficient to inhibit the folate metabolic pathways and derive the beneficial effects therefrom through administration of one or more of the pharmaceutical dosage units.

[0014] An exemplary daily dosage unit for a vertebrate host comprises an amount up to one gram of active compound per kilogram of the host, preferably one half gram, more preferably 100 milligrams, and most preferably, about 50 milligrams or less per kilogram of the host weight. The selected dose may be administered to a warm-blooded animal or mammal, for example, a human patient in need of treatment mediated by folate metabolic pathways inhibition, by any known method of administrating the dose including topically as, for example, an ointment or cream; orally; rectally, for example, as suppository; parenterally by injection; or continuously by intravaginal, intranasal, intrabronchial, intraaural or intraocular infusion.

[0015] The compounds according to the present invention may be characterized as producing any one or more of an antiproliferative effect, an antibacterial effect, an antiparasitic effect, an antiviral effect, an antipsoriatic effect, an antiprotozoal effect, an anticoccidial effect, an antiinflammatory effect, an immunosuppressive effect or an antifungal effect. The compounds are especially useful in producing an antitumor effect in a vertebrate host harboring a tumor.

[0016] The present invention relates to antiproliferative compounds capable of inhibiting GARFT and having the formula I



wherein:

n is 0 to 2;

A is sulfur, CH₂, oxygen, -NH- or selenium, provided, however, that when n is 0, A cannot be CH₂, and when n is 1, A cannot be CH₂ or NH;

X is at least one substituted or unsubstituted C₁-C₃ alkyl or alkenyl group, a C₂-C₃ alkynyl group, a substituted or unsubstituted amino group, sulfur or oxygen;

Ar is a substituted or unsubstituted monocyclic carbocyclic or heterocyclic ring or fused or nonfused carbocyclic or heterocyclic ring systems; and

R₁ and R₂ are independently hydrogen or a moiety which forms a readily hydrolyzable ester group or

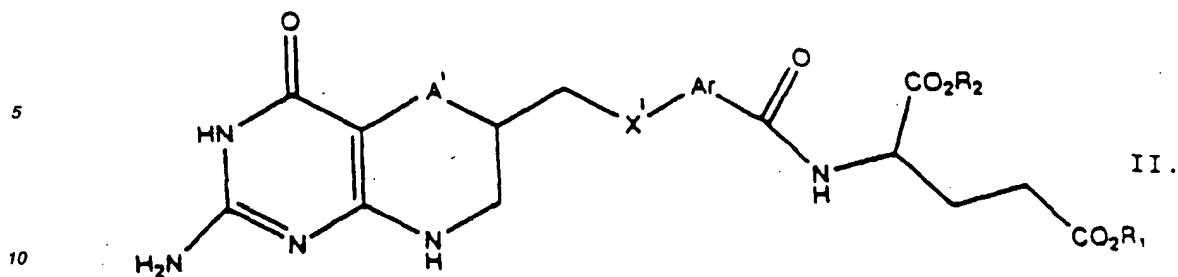
a pharmaceutically acceptable salt thereof.

[0017] Preferred moieties for R₁ and R₂ are hydrogen, C₁-C₆ alkyl, hydroxyalkyl, alkyaryl, and aralkyl. Particularly preferred moieties are hydrogen and C₂ alkyl.

[0018] Preferred substituents for X and Ar include C₁-C₆ alkyl or alkenyl, C₂-C₆ alkynyl, acyl, halogen, amino, hydroxyl, nitro, mercapto, monocyclic carbocyclic or heterocyclic rings, fused or nonfused carbocyclic or heterocyclic ring systems, hydroxyl or C₁-C₆ alkoxy C₁-C₆ alkyl.

[0019] It is particularly preferred that A be sulfur and that Ar be phenyl.

[0020] A preferred subgenus of the compounds of the invention has the formula II



wherein:

15 A' is sulfur or selenium;
 X' is CH_2 , sulfur, oxygen or NH ;
 Ar is as defined in formula I; and
 R_1 and R_2 are as defined in formula I or

20 a pharmaceutically acceptable salt thereof.

[0021] Preferred moieties for R_1 and R_2 are hydrogen, $\text{C}_1\text{-C}_6$ alkyl, hydroxyalkyl, alkyaryl, and aralkyl. Particularly preferred moieties are hydrogen and C_2 alkyl.

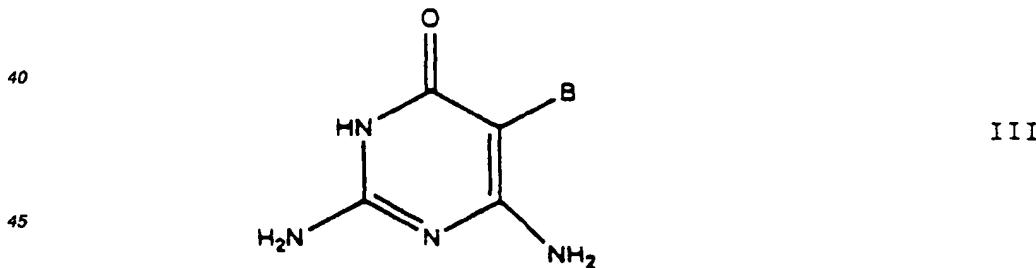
[0022] A' is preferably sulfur, X' is preferably CH_2 and Ar is preferably phenyl.

25 [0023] Although the compounds are depicted in the formulae I and II in the 4-oxo form and are referred to as such throughout this description, the oxo group exists in tautomeric equilibrium with the corresponding 4-hydroxy group and it will be understood that in each case the tautomeric hydroxyl form is also indicated.

[0024] The compounds of formulae I and II in which each of R_1 and R_2 is hydrogen are active anti-tumor and anti-proliferative compounds. The compounds of formula I wherein R_1 and R_2 are moieties which form a readily hydrolyzable ester group, preferably an ethyl group, are novel intermediates for forming the free glutamic acid forms of the compounds and can also be hydrolyzed *in vivo* and thus act as prodrugs.

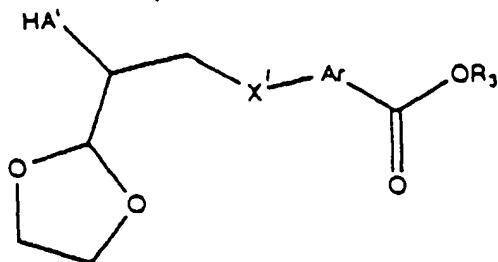
[0025] The invention also includes pharmaceutically acceptable salts, including, for example, alkaline metal, alkaline earth metal, other non-toxic metals, ammonium and substituted ammonium salts of the glutamic acid embodiments of the invention such as, but not limited to, the sodium, potassium, lithium, calcium, magnesium, aluminum, zinc, ammonium, trimethyl ammonium, triethyl ammonium, pyridinium and substituted pyridinium salts.

35 [0026] Novel compounds of this invention can be prepared by reacting a compound having the formula III

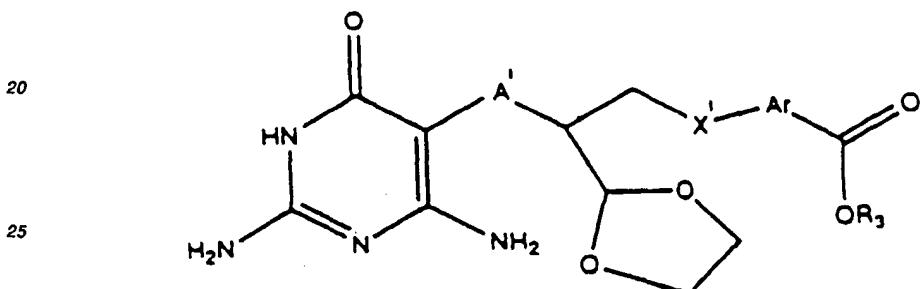


wherein:

50 B is a halogen, preferably bromo, with a compound having the formula IV



wherein X', A', and Ar are as defined in formula II and R₃ is hydrogen or a straight, branched or cyclic C₁ to C₆ alkyl group optionally carrying one or more halogen, hydroxyl, or amino groups; in the presence of base other than the compound of formula III, preferably a non-nucleophilic auxiliary base, in a solvent in which at least one of the reactants is at least partially soluble under conditions sufficient to obtain the compound of formula V



wherein A', X', and Ar are defined as in formula II and R₃ is as defined in formula IV.

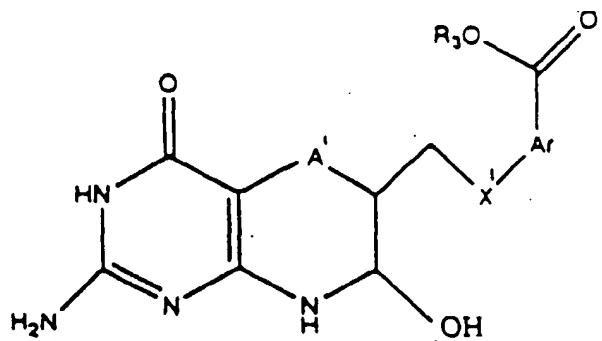
30 [0027] The reaction of compound III with compound IV is preferably carried out in a suitable solvent in which at least one or both reactants are soluble at the reaction temperature. The solvent and the reaction environment are preferably purged of oxygen prior to introduction of the reactants by bubbling an inert gas, such as argon or nitrogen, through the solvent. Bubbling of the inert gas is preferably continued until the reaction has gone to completion and been quenched, such as by pouring into water. Suitable preferred solvents are dipolar aprotic solvents such as, e.g., dimethylsulfoxide, N,N-dimethylformamide, N,N-dimethylacetamide, or N-methyl-2-pyrrolidinone.

35 [0028] The basic medium for the reaction of compounds III and IV is preferably provided via a non-nucleophilic auxiliary base which is defined as a base capable of neutralizing hydrogen halide gas generated by the substitution reaction. The base is preferably an alkali or alkali earth metal carbonate or a trialkylamine such as, e.g., trimethylamine, triethylamine or diisopropylethylamine.

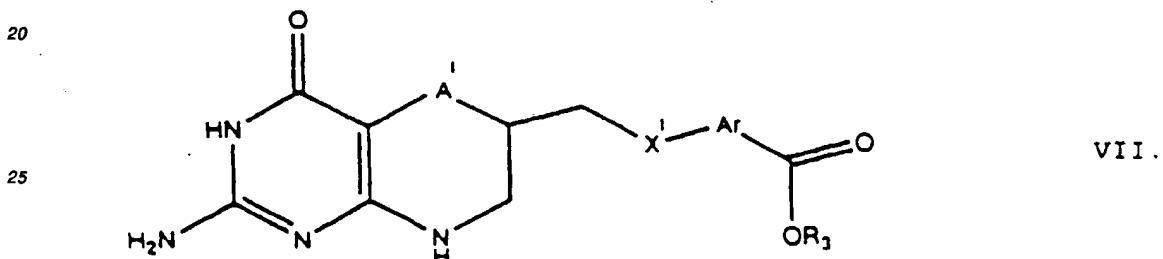
40 [0029] The preferred method for conducting the reaction of compounds of the formulae IV and III is to suspend the compound of formula III, preferably, 5-bromo-2,6-diamino-4(3H)-oxo-pyrimidine, in the solvent; compound of the formula IV and the auxiliary base are then added sequentially.

45 [0030] The reaction vessel can then immersed in an oil bath which has been heated to the appropriate temperature (20-200°, preferably 70-120°C). The reaction mixture can be stirred at this temperature for the requisite length of time (usually 30-330 minutes), then followed by cooling to room temperature and pouring into water. The product, the compound of formula V, can then be isolated by filtration or extraction with an organic solvent and purified either by recrystallization or by chromatography.

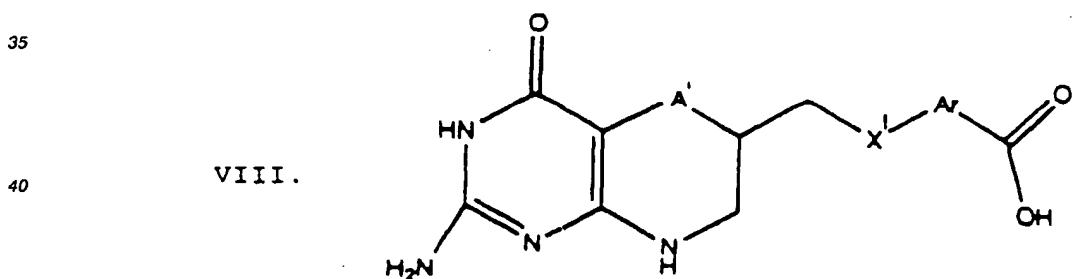
50 [0031] The compound of formula V can be reacted with an acid, preferably hydrochloric acid, in a suitable solvent, preferably tetrahydrofuran, under conditions sufficient, preferably reflux, to obtain a compound of the formula VI



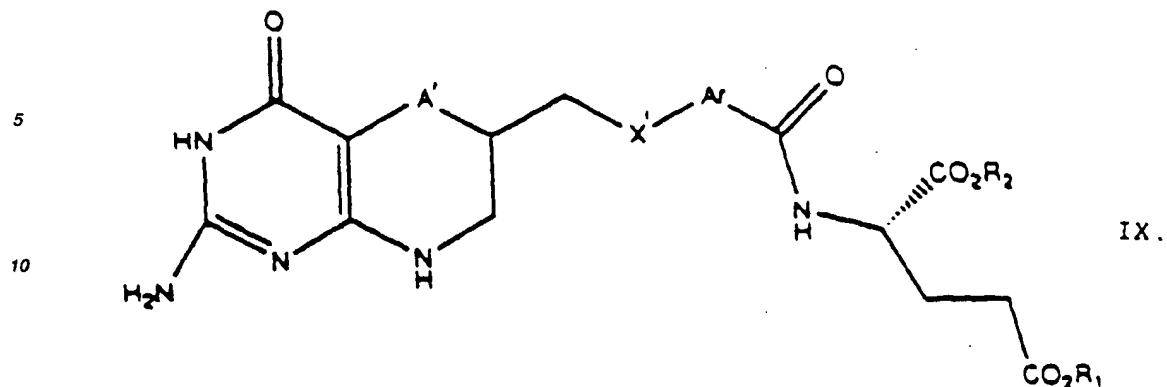
15 wherein A', X', and Ar are defined as in formula II and R₃ is as defined in formula IV.
 [0032] Compound VI is then reduced, preferably with sodium cyanoborohydride, to obtain a compound of the formula VII



30 wherein A', X', and Ar are defined as in formula II and R₃ is as defined in formula IV.
 [0033] The compound of formula VII is hydrolyzed, under basic conditions, to form a compound of the formula VIII



45 wherein A', X', and Ar are defined as in formula II. If, in the compound of formula VII, R₃ is hydrogen, then this reaction step is not necessary, and the compound of formula VII can be peptide coupled, as described immediately below.
 [0034] The compound of formula VIII (or the compound of formula VII, wherein R₃ is hydrogen), which is in free carboxylic acid form, can be peptide coupled, by means well-known to those skilled in the art, with a glutamic acid diester hydrochloride to form a diester of the formula IX



wherein A', X', Ar, R₁ and R₂ are as defined in formula II, with the proviso that neither R₁ nor R₂ is hydrogen.

[0035] Finally, if desired, the compound of formula IX can be hydrolyzed to the free glutamic acid form depicted in formula II (R₁ and R₂ = H). A detailed synthesis for compounds within formula II will be presented in the examples that follow.

20 [0036] Specific examples of novel compounds of formula II include:

(2-[4-[2-(2-Amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimido[5,4-b][1,4]thiazin-6-yl)-ethyl]-benzoylamino]pentanedioc acid) diethyl ester; and (2-[4-[2-(2-Amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimido[5,4-b][1,4]thiazin-6-yl)-ethyl]-benzoylamino]-pentanedioic acid). The d-form of these compounds is preferred.

25 [0037] It is to be understood that application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and a representative process for their preparation and recovery appear in the following examples.

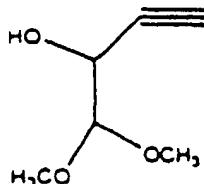
EXAMPLE 1

30

Step 1a

(1) (1,1-Dimethoxy-but-3-yn-2-ol)

35 [0038]



45

[0039] To a stirred solution of 1.037 g (10.55 mmol) of trimethylsilyl acetylene in 50 ml of dry tetrahydrofuran (THF) under argon at -78°C was added dropwise 6.6 ml of 1.6 M n-butyl lithium. After 10 minutes at -78°C, a solution of 1.21 g (10.46 mmol) of glyoxal dimethyl acetal in 5 ml of tetrahydrofuran (THF) was added dropwise. After 1 hour at -78°C, the reaction was quenched with about 1 ml of H₂O and allowed to warm to room temperature, diluted with ethyl acetate and washed with saturated NaCl solution. The aqueous layer was reextracted with ethyl acetate and combined organic layers were dried over MgSO₄ and concentrated under reduced pressure. To 785 mg of the resulting yellow oil dissolved in tetrahydrofuran (THF) was added 5.8 ml of 1.0 M tetrabutylammonium fluoride in tetrahydrofuran (THF). After heating for 1 hour at 50°C, the volatiles were evaporated and the residue was flash chromatographed on silica eluting methylene chloride/ethyl acetate (9:1). In this manner, there was obtained 412 mg (60% overall) of the alkyne-alcohol, compound (1), as a colorless oil.

IR (neat) 3441 (broad), 3277, 2944, 2839, 1636, 1450, 1196, 1084 cm⁻¹.

¹H NMR (CDCl₃) δ 2.42 (bs,1H), 2.49 (s,1H), 3.51 (s,3H), 3.53 (s,3H), 4.36 (bs,2H).

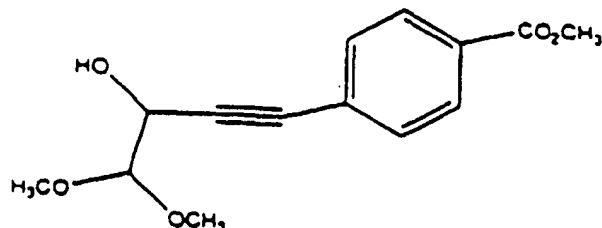
Analysis calculated for C₆H₁₀O₃ · 0.35 H₂O; C, 52.81; H, 7.90.

Found: C, 52.87; H, 7.86.

Step 1b

5 (2) (4-(3-Hydroxy-4,4-dimethoxy-but-1-ynyl)-benzoic acid methyl ester)

[0040]



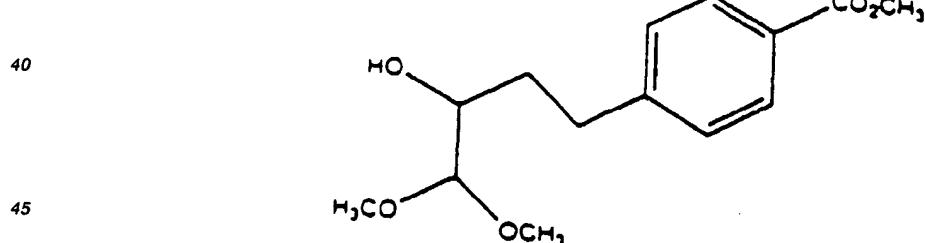
20 [0041] To a stirred solution of 232 mg (1.78 mmol) of the alkyne compound (1) and 467 mg (1.78 mmol) of Methyl-4-iodobenzoate in 5 ml diethylamine was added 13 mg (0.18 mmol) of Bis(triphenylphosphine) palladium(II)chloride and 7 mg (.036 mmol) of cuprous iodide. After 15 hours at room temperature, volatiles were removed under reduced pressure and the residue was flash chromatographed on silica eluting methylene chloride/ethyl acetate (12:1). In this manner, there was obtained 347 mg (74%) of the compound (2) as an orange oil.

25 IR (neat) 3451 (broad), 2953, 2838, 1717, 1607, 1437, 1310, 1283, 1120, 1082 cm⁻¹.
¹H NMR (CDCl₃) δ 2.46 (bs, 1H), 3.55 (s, 3H), 3.56 (s, 3H), 3.92 (s, 3H), 4.45 (d, 1H, J = 5.4 Hz), 4.60 (d, 1H, J = 5.3 Hz), 7.52 (d, 2H, J = 8.3 Hz), 7.98 (d, 2H, J = 8.3 Hz). Analysis Calculated for C₁₄H₁₆O₅ : C, 63.62; H, 6.10. Found: C, 63.14; H, 6.14.

30 Step 1c

[0042] (3) (4-(3-Hydroxy-4,4-dimethoxy-butyl)-benzoic acid methyl ester)

35 [0042]



50 [0043] A solution containing 14.37 g (54.38 mmol) of compound (2) and 1.40 g of 5% Pd on carbon in 175 ml of ethanol was hydrogenated under 40 psi Hydrogen on a Parr apparatus. After 2.5 hours, the reaction mixture was filtered and the catalyst washed with ethanol and methanol. After concentrating under reduced pressure, the residue was dissolved in methylene chloride and filtered through a short plug of silica, eluting methylene chloride, then methylene chloride/ethyl acetate (1:1) to remove the residual carbon. In this manner, there was obtained 14.34 g (98%) of the saturated alcohol, compound (3), as a yellow oil.

55 IR (neat) 3495 (broad), 2953, 1721, 1611, 1437, 1283, 1109, 1080 cm⁻¹.
¹H NMR (CDCl₃) δ 1.72-1.93 (m, 2H), 2.75-2.93 (m, 2H), 3.39 (s, 3H), 3.44 (s, 3H), 3.58 (m, 1H), 3.90 (s, 3H), 4.13 (d, 1H, J = 6.1 Hz), 7.29 (d, 2H, J = 8.1 Hz), 7.95 (d, 2H, J = 8.2 Hz). Analysis calculated for C₁₄H₂₀O₅ · 0.20 H₂O: C, 61.84; H, 7.56.

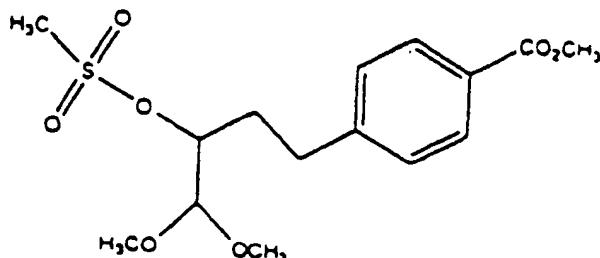
Found: C, 61.83; H, 757.

Step 1d (4)

5 (4-(3-Methanesulfonyloxy-4,4-dimethoxybutyl)-benzoic acid methyl ester)

[0044]

10



15

20 [0045] To a stirred solution of 206 mg (.77 mmol) of saturated alcohol, compound (3), and 0.16 ml (1.15 mmol) of triethylamine in 5 ml of methylene chloride at 0°C was added 0.07 ml (.85 mmol) of methanesulfonyl chloride. After 20 minutes at 0°C, another 0.02 ml of methanesulfonyl chloride was added. After 30 minutes more, the reaction mixture was poured into saturated NaHCO_3 solution and extracted twice with methylene chloride. The combined organic layers were dried over MgSO_4 and the solvent was removed under reduced pressure. This material was sufficiently pure for 25 use in the next step. An analytical sample was obtained by flash chromatography on silica eluting methylene chloride/ethyl acetate (20:1). In this manner, there was obtained the mesylate, compound (4), as a colorless oil.

IR (neat), 2949, 2839, 1719, 1611, 1437, 1352, 1283, 1177, 1109, 1078 cm^{-1} .

1H NMR (CDCl_3) δ 2.04 (m,2H), 2.77-2.91 (m,2H), 3.09 (s,3H), 3.41 (s,3H), 3.45 (s,3H), 3.90 (s,3H), 4.38 (d,1H, J = 5.5 Hz), 4.64 (m,1H), 7.29 (d,2H, J = 8.2 Hz), 7.96 (d,2H, J = 8.2Hz).

30 Analysis calculated for $\text{C}_{15}\text{H}_{22}\text{O}_7\text{S}$: C, 52.01; H, 6.40; S, 9.26

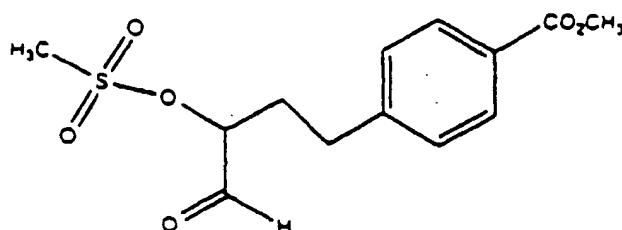
Found: C, 52.08; H, 6.44; S, 9.25.

Step 1e

35 (5) (4-(3-Methanesulfonyloxy-4-oxobutyl)-benzoic acid methyl ester)

[0046]

40



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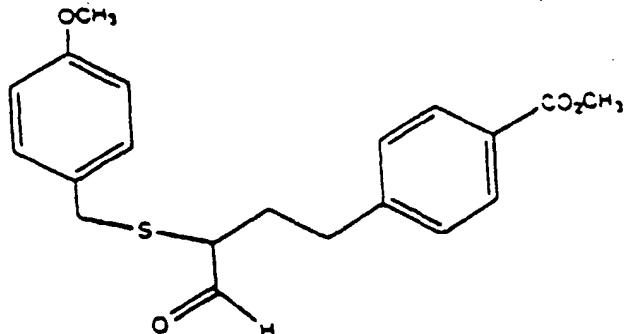
50 [0047] To a stirred solution of 600 mg (1.73 mmol) of dimethyl acetal (mesylate) (compound 4) in 5 ml of methyl chloride at 0°C was added 1 ml of H_2O and 1 ml of Trifluoroacetic acid. The reaction was warmed to room temperature, then refluxed for 24 hours. The cooled reaction mixture was diluted with ethyl acetate and washed twice sequentially with saturated NaCl solution, saturated NaHCO_3 solution, then again with saturated NaCl solution, dried (MgSO_4) and the volatiles were removed under reduced pressure. In this manner, there was obtained the mesylate, compound (5), 55 which was used without purification.

NMR (CDCl_3) δ 2.20 (m,2H), 2.85 (m,2H), 3.17 (s,3H), 3.91 (s,3H), 4.95 (dd,1H, J = 4.2 Hz, 8.4 Hz), 7.29 (d,2H, J = 8.1 Hz), 7.99 (d,2H, J = 8.2 Hz), 9.59 (s,1H).

Step 1f

(6) (4-[3-(4-Methoxy-benzylsulfanyl)-4-oxobutyl]-benzoic acid methyl ester

5 [0048]



[0049] To a stirred solution of 686 mg (2.28 mmol) of the mesylate, compound (5), and 0.40 ml (2.29 mmol) of N,N-Diisopropylethylamine in dimethyl formamide (DMF) was added 0.48 ml (3.44 mmol) of 4-Methoxy- α -toluenethiol. After 3 hours at room temperature, the reaction mixture was poured into 0.5 N HCl and extracted twice with ethyl acetate. The combined organic layers were washed twice with saturated NaCl solution, dried (MgSO_4), and concentrated under reduced pressure. The aldehyde obtained, compound (6), was sufficiently pure to use without further purification in the next step.

IR (KBr) 2930, 1715, 1703, 1611, 1512, 1282, 1244, 1107 cm^{-1} .

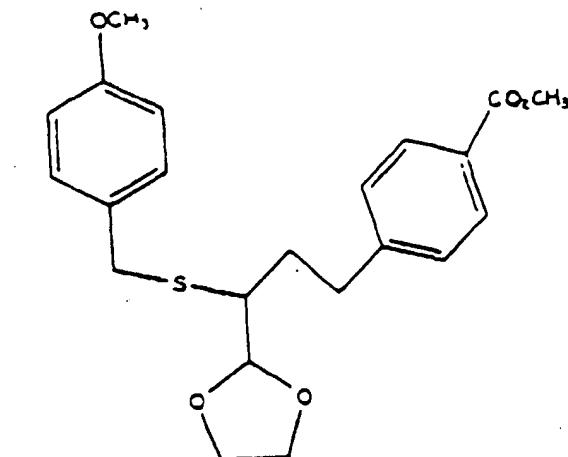
^1H NMR (CDCl_3) δ 1.80-2.16 (m,2H), 2.75 (m,2H), 2.99 (m,1H), 3.53 (AB,2H, J = 13.4 Hz), 3.81 (s,3H), 3.90 (s,3H), 6.83 (d,2H, J = 8.5 Hz), 7.12 (d,2H, J = 8.2 Hz), 7.19 (d,2H, J = 8.6 Hz), 7.90 (d,2H, J = 8.2 Hz), 9.27 (d,1H, J = 4.2 Hz).

30

Step 1g

(7) (4-[3-[1,3]Dioxolan-2-yl-3-(4-methoxy-benzylsulfanyl)propyl]-benzoic acid methyl ester

35 [0050]



[0051] A flask containing 301 mg (.84 mmol) of the aldehyde, compound (6), 94 μl (1.68 mmol) of ethylene glycol and 42 mg (.17 mmol) of Pyridinium p-toluenesulfonate and 30 ml of benzene was heated at reflux removing the generated water with a Dean-Stark trap. After 3 hours, the reaction mixture was poured into saturated NaCl solution

and extracted twice with ethyl acetate. The combined organic layers were dried (MgSO_4) and concentrated under reduced pressure. The residue was flash chromatographed on silica, eluting hexanes-ethyl acetate (5:1). In this manner, there was obtained the title compound (7) in an overall 84% yield from the mesylate dimethylacetal, compound (4).
 5 IR (neat), 2949, 2886, 1721, 1611, 1510, 1435, 1279, 1248, 1177, 1111, 1034 cm^{-1} .

^1H NMR (CDCl_3) δ 1.67 (m, 1H), 1.99 (m, 1H), 2.57 (m, 2H), 2.84 (m, 1H), 3.72-4.03 (m, 6H), 3.81 (s, 3H), 3.90 (s, 3H), 4.97 (d, 1H, J = 4.6 Hz), 6.83 (d, 2H, J = 8.6 Hz), 7.09 (d, 2H, J = 8.1 Hz), 7.23 (d, 2H, J = 8.5 Hz), 7.88 (d, 2H, J = 8.1 Hz).
 Analysis calculated for $\text{C}_{22}\text{H}_{26}\text{O}_5\text{S}$: C, 65.65; H, 6.51; S, 7.97.
 Found: C, 65.72; H, 6.50; S, 8.07.

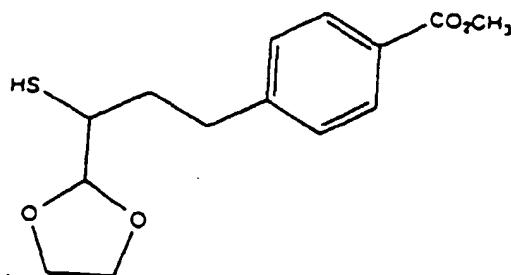
10 Step 1h

(8) (4-(3-[1,3]Dioxolan-2-yl-3-mercaptopropyl)benzoic acid methyl ester)

[0052]

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[0053] To a stirred solution of 5.70 g (14.16 mmol) of compound (7) and 5.42 g (17.00 mmol) of Mercuric acetate in methylene chloride, cooled to 0°C, was added, dropwise, 5 ml of trifluoroacetic acid. After 3 hours at 0°C, hydrogen sulfide saturated methanol was added and stirring was continued at 0°C for 20 minutes. The reaction mixture was poured into saturated NaCl solution and extracted twice with methylene chloride. The combined organic layers were dried (MgSO_4) and concentrated at reduced pressure. To the crude residue partially suspended in methanol was added 1.07 g (28.28 mmol) of sodium borohydride in portions. After about 30 minutes, the reaction mixture was poured into ethyl acetate and 0.5 N HCl and the layers separated. The metallic mercury that formed in the reaction was washed with ethyl acetate. The combined organic layers were washed with saturated NaCl solution, dried (MgSO_4) and concentrated under reduced pressure. The residue was flash chromatographed on silica eluting Hexanes - ethyl acetate (4:1). In this manner, there was obtained 1.99 g (50%) of the thiol, compound (8), as a light yellow oil.
 IR (neat), 2951, 2886, 1719, 1611, 1435, 1281, 1179, 1144, 1111 cm^{-1} .
 ^1H NMR (CDCl_3) δ 1.64 (d, 1H, J = 7.8 Hz), 1.74 (m, 1H), 2.15 (m, 1H), 2.80 (m, 2H), 3.00 (m, 1H), 3.90 (s, 3H), 3.97 (m, 4H), 4.91 (d, 1H, J = 4.0 Hz), 7.28 (d, 2H, J = 8.1 Hz), 7.95 (d, 2H, J = 8.1 Hz).
 Analysis calculated for $\text{C}_{14}\text{H}_{18}\text{O}_4\text{S}$: C, 59.55; H, 6.14. Found: C, 59.42; H, 6.41.

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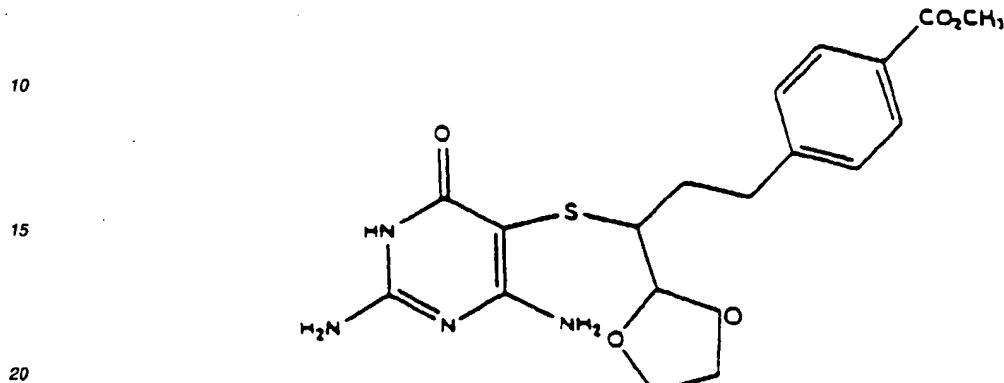
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Step 1i

(9) (4-[3-(2,4-diamino-6-oxo-1,6-dihydro-pyrimidin-5-ylsulfanyl)-3-[1,3]dioxolan-2-yl-propyl]-benzoic acid methyl ester)

5 [0054]



25 [0055] To a stirred solution of 1.91 g (6.76 mmol) of the thiol, compound (8), and 1.39 g (6.78 mmol) of 5-bromo-2,4-diamino-6-oxopyrimidine under argon in degassed N,N-Dimethylformamide was added 1.18 ml (6.77 mmol) of N,N-Diisopropylethylamine. The reaction mixture was heated at 90°C for 3 hours. The cooled reaction mixture was poured into saturated NaCl solution and the precipitate which formed was collected by filtration, washed with water and air dried. The filter cake was slurried in methylene chloride. Hexanes were slowly added and the precipitate was again collected by filtration, washed with hexanes and dried. In this manner, there was obtained 1.91 g (69 %) of the desired dioxolane, compound (9), as an off-white solid: mp 206-208°C with decomposition.

30 IR (KBr) 3439, 3341, 3154, 1701, 1636, 1591, 1470, 1447, 1287 cm⁻¹.

¹H NMR (DMSO) δ 1.64 (m, 1H), 1.84 (m, 1H), 2.58 (m, 1H), 2.79 (m, 1H), 3.19 (m, 1H), 3.82 (s, 3H), 3.85 (m, 4H), 4.83 (d, 1H, J = 4.3 Hz), 6.33 (broad s, 4H), 7.33 (d, 2H, J = 8.1 Hz), 7.83 (d, 2H, J = 8.1 Hz), 10.03 (s, 1H).

Analysis calculated for C₁₈H₂₂N₄O₅S: C, 53.19; H, 5.46; N, 13.78; S, 7.89.

Found: C, 52.98; H, 5.53; N, 13.60; S, 7.76.

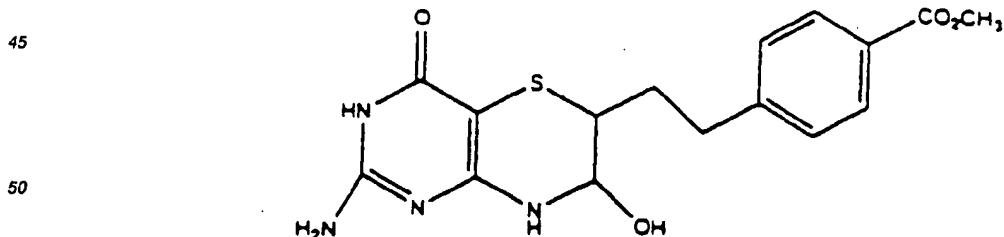
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Step 1j

(10) (4-[2-(2-Amino-7-hydroxy-4-oxo-4,6,7,8-tetrahydro-3H-pyrimido[5,4-b][1,4]thiazin-6-yl)-ethyl]-benzoic acid methyl ester)

40

[0056]



55 [0057] A stirred suspension of 1.20 g (2.85 mmol) of the dioxolane, compound (9), and 4 ml of 2 N HCl in 20 ml of tetrahydrofuran (THF) was heated at reflux for 2.5 hours. The homogeneous solution was poured slowly into saturated NaHCO₃ solution and the precipitate that formed was collected. The filtrate was extracted with ethyl acetate. A precipitate (58 mg) formed between layers and was collected and combined with the first precipitate. The ethyl acetate layer was dried over MgSO₄ and the solvent was removed under reduced pressure. The residue (42 mg) was also

combined with the original precipitate. In this manner, there was obtained 984 mg (98%) of the carbinolamine, compound (10), as an orange solid: mp 213-216°C.

IR (KBr) 3351, 3441, 1705, 1638, 1609, 1557, 1470, 1289, 1113, 1020 cm^{-1} .

5 ^1H NMR As a single pair of diastereomers (DMSO) δ 1.39 and 1.96 (m,m,1H), 1.70 (m,1H), 2.56-2.89 (m,3H), 3.82 (s,3H), 4.71 and 4.84 (m,m,1H), 5.37 and 5.40 (d,d,1H,J = 6.6 Hz), 6.06 (s,2H), 7.20 (d,1H,J = 4.5 Hz), 7.31 and 7.36 (d,d,2H,J = 8.1 Hz), 7.86 and 7.88 (d,d,2H,J = 8.0 Hz), 10.16 and 10.19 (s,s,1H).

Analysis calculated for $\text{C}_{16}\text{H}_{18}\text{N}_4\text{O}_4\text{S} \cdot 1.7 \text{ H}_2\text{O}$: C, 48.89; H, 5.49; N, 14.26; S, 8.16.

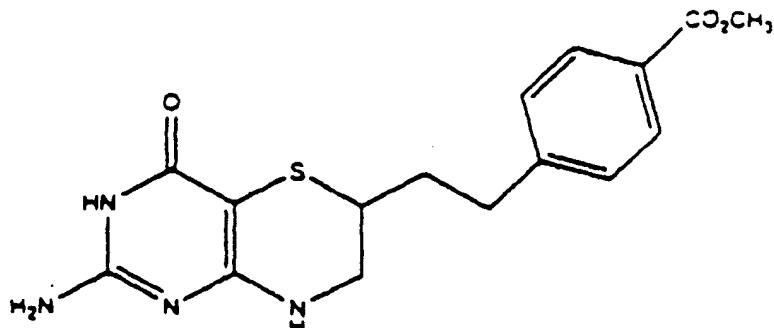
Found: C, 48.78; H, 5.18; N, 14.00; S, 8.03.

10 Step 1k

(11) (4-[2-(Amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimido[5,4

15 b][1,4]thiazin-6-yl]-benzoic acid methyl ester

[0058]



[0059] To a 0°C suspension of 1.126 g (3.1 mmol) of carbinolamine, compound (10), in tetrahydrofuran (THF) was added 2.3 ml (18.64 mmol) of Boron trifluoride etherate. When the addition was complete, 0.586 g (9.32 mmol) of Sodium cyanoborohydride was added in portions over 5 minutes. After an additional 30 minutes, 5 ml of ammonia saturated methanol was added, the reaction mixture was diluted with ethyl acetate and washed with saturated NaCl solution. The organic layer was dried (MgSO_4) and the solvent removed under reduced pressure. The residue was flash chromatographed on silica eluting methylene chloride/ methanol (9:1). In this manner, there was obtained 542 mg (50%) of the dehydrated ester, compound (11), as an orange solid: mp 245-246°C with decomposition.

IR (KBr) 3358, 2936, 1721, 1644, 1595, 1537, 1447, 1346, 1281 cm^{-1} .

40 ^1H NMR (DMSO) δ 1.72 (m,1H), 1.90 (m,1H), 2.80 (m,3H), 3.22 (m,1H), 3.52 (m,1H), 3.82 (s,3H), 6.00 (s,2H), 6.65 (s,1H), 7.37 (d,2H,J = 8.1 Hz), 7.87 (d,2H,J = 8.1 Hz), 10.05 (s,1H).

Analysis calculated for $\text{C}_{16}\text{H}_{18}\text{N}_4\text{O}_3\text{S}$: C, 55.47; H, 5.24; N, 16.17; S, 9.26.

Found: C, 55.31; H, 5.29; N, 16.09; S, 9.17.

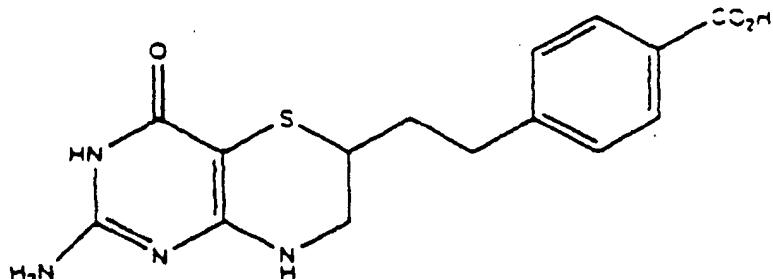
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Step 1(12) (4-[2-(2-Amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimido [5,4-b][1,4]thiazin-6-yl)-ethyl]-benzoic acid)

5 [0060]



10 [0061] A solution of 530 mg (1.53 mmol) of the ester, compound (11), and 10 ml of 1N NaOH was stirred at room
 20 temperature for 30 minutes. The homogeneous solution was made slightly acidic (pH 4) with concentration HCl. After cooling in an ice bath, the light orange precipitate was collected by filtration and air dried. It was then suspended in ethanol and the ethanol removed under reduced pressure. In this manner, there was obtained 468 mg (91%) of the acid, compound (12): decomposes > 310°C.

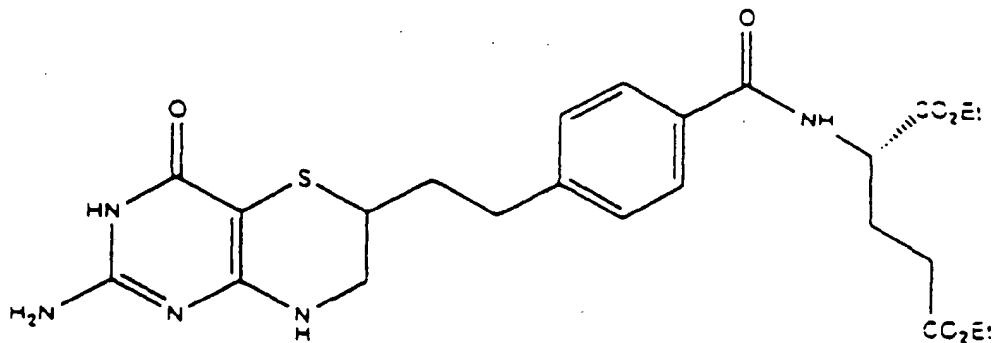
25 IR (KBr) 3285, 3086, 2928, 1698, 1642, 1611, 1576, 1449, 1348 cm^{-1} .

30 ^1H NMR (DMSO) δ 1.72 (m, 1H), 1.89 (m, 1H), 2.78 (m, 3H), 3.20 (m, 1H), 3.48 (m, 1H), 6.07 (s, 2H), 6.68 (s, 1H), 7.33 (d, 2H, J = 8.1 Hz), 7.85 (d, 2H, J = 8.1 Hz), 10.11 (s, 1H), 12.77 (broad s, 1H).

35 Analysis calculated for $\text{C}_{15}\text{H}_{16}\text{N}_4\text{O}_3\text{S} \cdot 1.20 \text{ H}_2\text{O}$: C, 50.89; H, 5.24; N, 15.83; S, 9.06.
 Found: C, 50.70; H, 4.92; N, 15.58; S, 8.87.

30 Step 1m(13) (2-[4-[2-(2-Amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimido [5,4-b][1,4]thiazin-6-yl)-ethyl]-benzoylamino]-pentanedioic acid diethyl ester

35 [0062]



40 [0063] To a stirred solution of 397 mg (1.19 mmol) of the acid, compound (12), 169 mg (1.25 mmol) of 1-Hydroxy-benzotriazole hydrate (HOBT), .22 ml (1.25 mmol) of N,N-Diisopropylethylamine and 300 mg (1.25 mmol) of L-Glutamic acid diethyl ester hydrochloride in 15 ml of N,N-dimethylformamide was added 240 mg (1.25 mmol) of 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC). After 18 hours at room temperature, the reaction mixture was
 45 poured into ice cold saturated NaCl solution and the precipitate which formed was collected, washed with H_2O and air dried. The precipitate was flash chromatographed on silica eluting methylene chloride/ methanol (9:1). In this manner, there was obtained 357 mg (58%) of the desired product (13) as a light orange solid: mp 132-136°C.
 50 IR (KBr) 3333, 1732, 1645, 1572, 1535, 1449, 1343, 1203, 1020 cm^{-1} .

1H NMR (DMSO) δ 1.15 (t,3H,J = 7.3 Hz), 1.17 (t,3H,J = 7.3 Hz), 1.72 (m,1H), 1.88-2.10 (m,3H), 2.42 (t,2H,J = 7.4 Hz), 2.79 (m,3H), 3.22 (m,1H), 3.50 (m,1H), 4.02 (q,2H,J = 7.3; 14.5Hz), 4.09 (q,2H,J = 7.2, 14.3 Hz), 4.41 (m,1H), 6.21 (s,2H), 6.74 (s,1H), 7.32 (d,2H,J = 8.0 Hz), 7.80 (d,2H,J = 8.0 Hz), 8.64 (d,1H,J = 7.41 Hz), 10.24 (s,1H)

5 Analysis calculated for $C_{24}H_{31}N_5O_6S$: C, 55.69; H, 6.04; N, 13.53; S, 6.19.
Found: C, 55.41; H, 6.11; N, 13.48; S, 6.12.

Step 1n

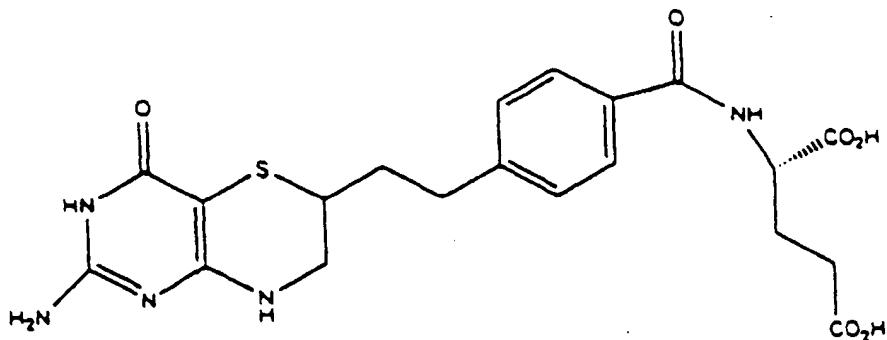
10 (14) (2-[4-{2-(2-Amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimido[5,4-b][1,4]thiazin-6-yl)-ethyl]benzoyl amino)pentanedioic acid)

[0064]

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30 [0065] A mixture of 320 mg (.618 mmol) of glutamate, compound (13), and 6 ml of 1N NaOH was stirred at room temperature for 3 hours, neutralized with concentrated HCl, then made slightly acidic with 2N HCl. After cooling, the light yellow precipitate was collected and air dried. The filter cake was taken up in ethanol/acetonitrile and any residual H₂O was azeotrophed off. In this manner, there was obtained 220 mg (77%) of the diacid, compound (14): mp: 188-190°C.

35 IR (KBr) 3348 (broad), 2930, 1717, 1642, 1539, 1505, 1348 cm⁻¹.
1H NMR (DMSO) δ 1.71 (m,1H), 1.92 (m,2H), 2.08 (m,1H), 2.34 (t,2H,J = 7.4 Hz), 2.79 (m,3H), 3.20 (m,1H), 3.55 (m, 1H), 4.38 (m,1H), 6.07 (s,2H), 6.68 (s,1H), 7.31 (d,2H,J = 8.1 Hz), 7.80 (d,2H,J = 8.2 Hz), 8.53 (d,1H,J = 7.7 Hz), 10.11 (s,1H), 12.40 (broad s,2H).

Analysis calculated for $C_{20}H_{23}N_5O_6S \cdot 1.5 H_2O$: C, 49.17; H, 5.36; N, 14.34; S, 6.56.
Found: C, 48.77; H, 4.97, N, 14.07; S, 6.54.

40

EXAMPLE 2

Biological and Biochemical Evaluation

45 In Vitro Testing

50 [0066] Cellular growth in the presence of the compounds according to the present invention was assessed using the L1210 murine leukemia (ATCC CCL 219) cell line. The cell line was maintained in RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum without antibiotics.

55 [0067] IC₅₀ values were determined in 160 microliter microcultures containing 1500 (L1210) cells established in 96 well plates in growth medium supplemented with 50 IU/mL penicillin and 50 mcg/mL streptomycin. Growth was measured over 3 days of continuous exposure to varying concentrations of the test compound (14) added 4 hours after initial cell plating by the MITT-tetrazolium reduction assay of Mosmann (Immunol. Meth. 65, 55-63 (1983)), modified according to Alley et al. (Cancer Res. 48, 589-601 (1988)). Water insoluble derivatives were dissolved in DMSO and diluted to a final concentration of 0.5% solvent in cell cultures.

Determination of Inhibition Constants for Gar Transformylase

[0068] GAR transformylase inhibition constant was measured by the method of Cleland (Biochem. Biophys. Acta 67, 173-187 (1963)). Assays were done at 22°C and initiated by addition of enzyme using the spectrophotometric assay of Young et al. (Biochemistry 23, 3979-3986 (1984)) and monitoring the reaction at 295 nm. The GAR transformylase domain of the human enzyme was used. The variable substrate was 10-formyl-5,8-dideazafolate at concentrations of 0.83 μ M, 1.25 μ M, 2.5 μ M and 5 μ M while the other substrate, GAR (glycinamide ribonucleotide), was held constant at 20 μ M. The assay mix contained 20 mM Hepes pH 7.5, 20 μ M GAR, and variable amounts of 10-formyl-5, 6-dideazafolate and inhibitor. For the inhibitor (14), five concentrations were used ranging from 0 to approximately 3K_i. The data were plotted as the velocity of the reaction versus the reciprocal of the 10-formyl-5, 8-dideaza-folate concentration. The inhibition constant was measured from a replot of the slopes of these lines obtained for each concentration of inhibitor versus the inhibitor concentration.

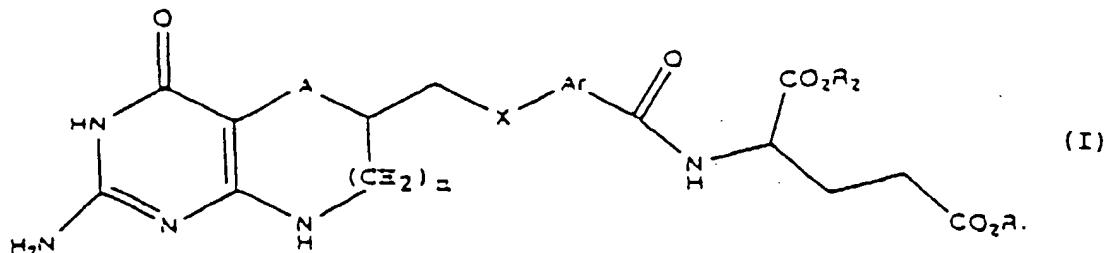
GARFT Inhibition and Cell Culture Data			
Num.	GARFT K _i μ M	L1210	Cell Line, IC ₅₀ (μ M)
(14)	0.035	0.05	

[0069] The compounds and intermediates of the invention contain one or more chiral centers. The invention encompasses racemic mixtures, mixtures of diastereomers, and optically active compounds, such as compounds essentially free of other optical isomers, which optically active compounds can be obtained by means well-known to those skilled in the art.

[0070] It will be apparent to those skilled in the art that various modifications and variations can be made in the processes and products of the present invention. Thus, it is intended that the present invention cover modifications and variations of this invention which fall within the scope of the appended and equivalent claims.

Claims

1. A compound having the formula (I)



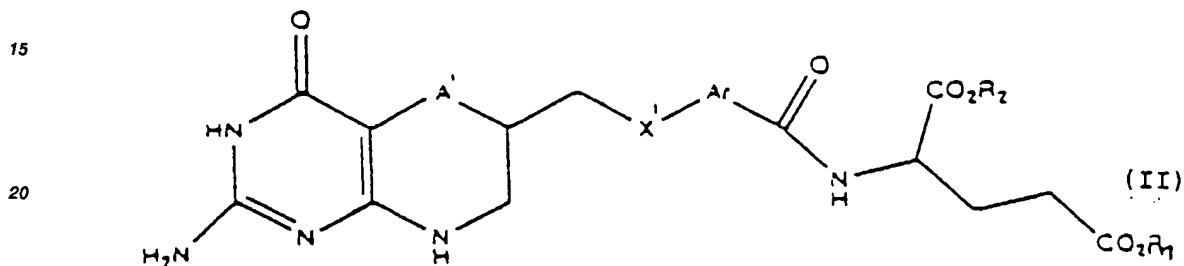
45 wherein

n is 0 to 2;
A is sulfur, CH₂, oxygen, -NH- or selenium, provided that when n is 0, A cannot be CH₂, and when n is 1, A cannot be CH₂ or NH;
50 X is at least one substituted or unsubstituted C₁-C₃ alkyl or alkenyl group, a C₂-C₃ alkynyl group, a substituted or unsubstituted amino group, sulfur or oxygen;
Ar is a substituted or unsubstituted monocyclic carbocyclic or heterocyclic ring or fused or nonfused carbocyclic or heterocyclic ring system; and
55 R₁ and R₂ are independently hydrogen or a moiety which forms a readily hydrolyzable ester group or a pharmaceutically acceptable salt thereof.

2. The compound or salt according to claim 1, wherein R₁ and R₂ are independently hydrogen, C₁-C₆ alkyl, hydroxy-

alkyl, alkylaryl, and aralkyl.

3. The compound or salt according to claim 2, wherein R₁ and R₂ are independently hydrogen and C₂ alkyl.
- 5 4. The compound or salt according to claim 1, wherein the substituents for X and Ar are selected from the group C₁-C₆ alkyl or alkenyl, C₂-C₆ alkynyl, acyl, halogen, amino, hydroxyl, nitro, mercapto, monocyclic carbocyclic or heterocyclic rings, fused or nonfused carbocyclic or heterocyclic ring systems, hydroxyl and C₁-C₆ alkoxy C₁-C₆ alkyl.
- 10 5. The compound or salt according to claim 1, wherein A is sulfur and Ar is phenyl.
6. A compound of the formula (II)



25 wherein:

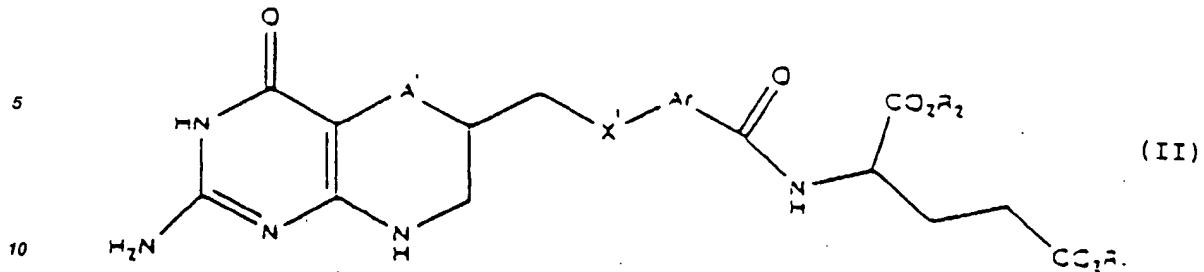
A' is sulfur or selenium;

X' is CH₂, sulfur, oxygen or NH;

30 Ar is a substituted or unsubstituted monocyclic carbocyclic or heterocyclic ring or fused or nonfused carbocyclic or heterocyclic ring system; and

R₁ and R₂ are independently hydrogen or a moiety which forms a readily hydrolyzable ester group or a pharmaceutically acceptable salt thereof.

- 35 7. The compound or salt according to claim 6 wherein R₁ and R₂ are independently hydrogen, C₁-C₆ alkyl, hydroxyalkyl, alkylaryl, and aralkyl.
8. The compound or salt according to claim 7, wherein R₁ and R₂ are independently hydrogen and C₂ alkyl.
- 40 9. The compound or salt according to claim 6, wherein A' is sulfur, X' is CH₂ and Ar is phenyl.
10. An antiproliferative composition comprising the compound or salt according to any of claims 1 to 9 in combination with a pharmaceutically acceptable carrier.
- 45 11. Use of the compound or salt according to any of claims 1 to 9 for the preparation of a medicament for inhibiting the growth and proliferation of the cells of microorganisms or of higher organisms in a host.
12. Use of the compound or salt according to any of claims 1 to 9 for the preparation of a medicament for inhibiting GRAFT.
- 50 13. A process for preparing a compound of the formula (II)



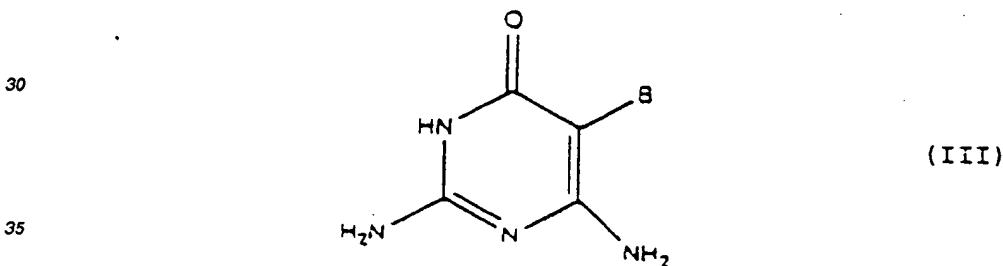
wherein:

15 A' is sulfur or selenium;
 X' is CH₂, sulfur, oxygen or NH;
 Ar is a substituted or unsubstituted monocyclic carbocyclic or heterocyclic ring or fused or nonfused carbocyclic or heterocyclic ring system; and

20 R₁ and R₂ are independently hydrogen or a moiety which forms a readily hydrolyzable ester group, with the proviso that neither R₁ nor R₂ is hydrogen; or
 a pharmaceutically acceptable salt thereof;

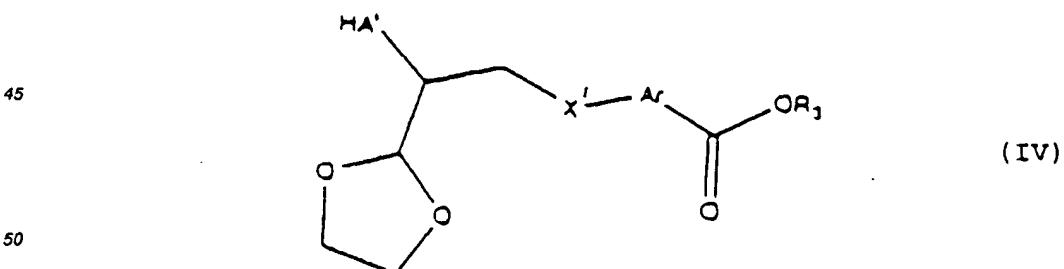
which process comprises the steps of

25 (a) reacting a compound having the formula (III)



wherein:

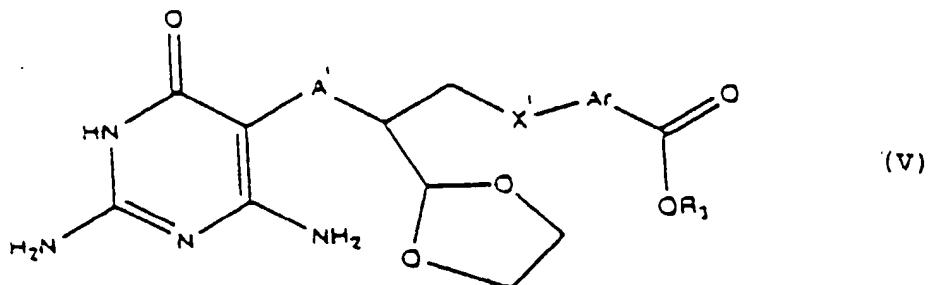
40 B is a halogen with a compound having the formula (IV)



55 wherein X', A', and Ar are as defined in formula II and R₃ is hydrogen or a straight, branched or cyclic C₁ to C₆ alkyl group optionally carrying one or more halogen, hydroxyl, or amino groups; in the presence of base other than the compound of formula (III) in a solvent in which at least one of the reactants is at least partially soluble under conditions sufficient to obtain the compound of formula (V)

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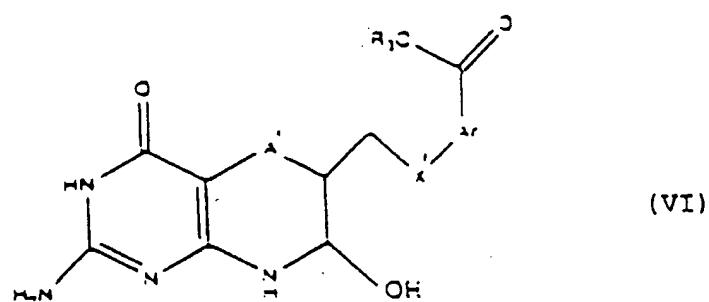


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wherein A', X', and Ar are defined as in formula (II) and R₃ is as defined in formula (IV);
 (b) reacting the compound of formula (V) with an acid in a suitable solvent under conditions sufficient to obtain a compound of the formula (VI)

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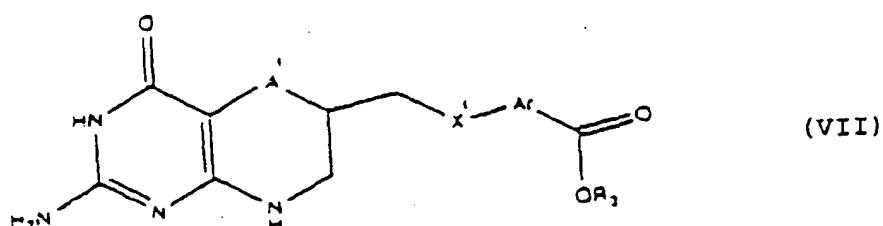


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wherein A', X', and Ar are defined as in formula II and R₃ is as defined in formula (IV);
 (c) reducing the compound of formula VI under conditions sufficient to obtain a compound of the formula (VII)

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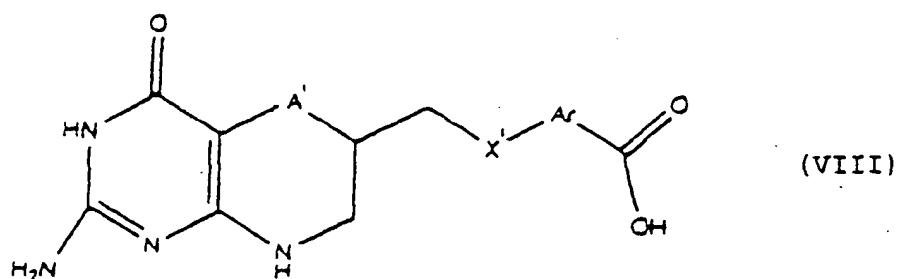


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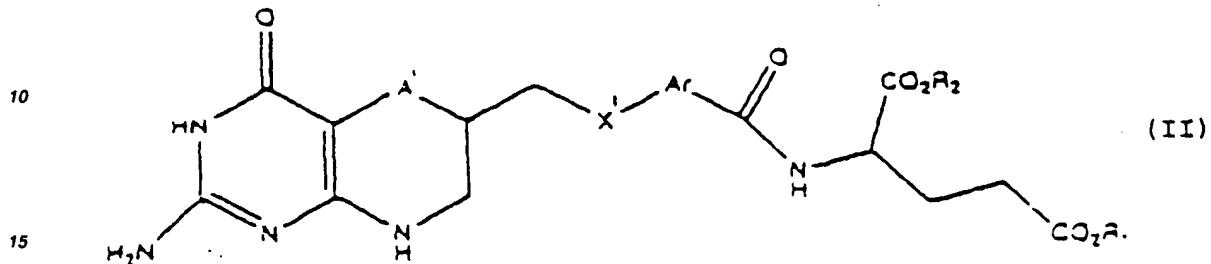
wherein A', X', and Ar are defined as in formula (II) and R₃ is as defined in formula (IV);
 (d) hydrolyzing the compound of formula (VII), in the case where R₃ in the compound of formula (VII) is not hydrogen, under basic conditions sufficient to form a compound of the formula (VIII)

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55



5
 wherein A', X', and Ar are defined as in formula (II); and
 (e) peptide coupling the compound of formula (VIII) or the compound of formula (VII), wherein R₃ is hydrogen, with a glutamic acid diester hydrochloride to form a diester of the formula (II), as defined above, with the proviso that neither R₁ nor R₂ is hydrogen.



20
 14. The process of claim 13, further comprising the step of hydrolyzing the compound of formula (II) to the free glutamic acid form depicted in formula (II), wherein both R₁ and R₂ are hydrogen.

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 15. The process of claim 13, wherein in step (a), B is bromine.

16. The process of claim 15, wherein in step (a), the base other than the compound of formula III is a non-nucleophilic auxiliary base.

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 17. The process of claim 16, wherein in step (b), the acid is hydrochloric acid.

18. The process of claim 17, wherein in step (b), the solvent is tetrahydrofuran.

19. The process of claim 18, wherein in step (c) the reducing is accomplished by the use of sodium cyano borohydride.

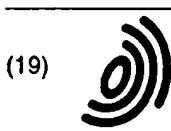
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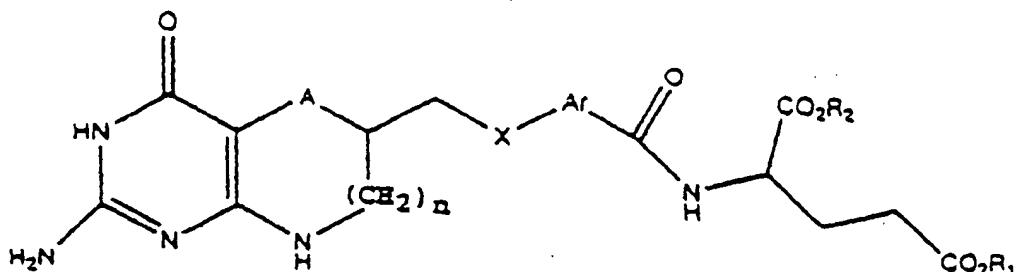
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(54) Condensed heterocyclic glutamic acid derivatives as antiproliferative agents

(57) Novel compounds of formula (I)



wherein

n is 0 to 2;

A is sulfur, CH₂, oxygen, -NH- or selenium, provided that when n is 0, A cannot be CH₂, and when n is 1, A cannot be CH₂ or NH;X is at least one substituted or unsubstituted C₁-C₃ alkyl or alkenyl group;a C₂-C₃ alkynyl group, a substituted or unsubstituted amino group, sulfur or oxygen;

Ar is a substituted or unsubstituted monocyclic carbocyclic or heterocyclic ring or fused or nonfused carbocyclic or heterocyclic ring system; and

R₁ and R₂ are independently hydrogen or a moiety which forms a readily hydrolyzable ester group or a pharmaceutically acceptable salt thereof
are found to inhibit the enzyme glycinamide ribonucleotide formyl transferase (GARFT).

A novel method of preparing such compounds is also disclosed, as well as methods and compositions for employing

the compounds as antiproliferative agents.



European Patent
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EUROPEAN SEARCH REPORT

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Place of search		Date of completion of the search	Examiner
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ANNEX TO THE EUROPEAN SEARCH REPORT
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